



Effect of chrysin endothelial vasodilation on L-NAME induced hypertensive rats

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Hypertension is the major risk factor for cardiovascular diseases and stroke and it is one of leading causes of disability and mortality worldwide. The present study was aimed to investigate the antihypertensive and vasodilation properties of chrysin, a naturally occurring flavone, in hypertensive rats. Hypertension was induced using N^o-nitro-L arginine methyl ester (L-NAME; 40 mg/kg BWT/day for 4 weeks) in adult rats and chrysin (25 mg/kg BWT/day) or vehicle was administered for 4 weeks. A significant increase of blood pressure and serum fibrinogen along with a significant reduction of bilirubin level were observed in hypertensive rats and these changes were significantly attenuated upon administration of chrysin. Further, L-NAME induced increase of tissue levels of mRNAs of β -MHC and TGF- β (in heart) as well as eNOS (in aorta, heart, kidney) were significantly reduced in chrysin treated rats. These results suggest that chrysin has a potential to attenuate impairment of organs (heart and renal) and endothelial function caused by L-NAME induced hypertension.

Keywords: β -MHC, Chrysin, Fibrinogen, mRNA expression, Nitric oxide, TGF- β

Hypertension is one of the most important factors associated with development of several diseases such as heart failure, renal failure, coronary heart disease, atherosclerosis, myocardial infarction and stroke. Endothelial dysfunction, which results from nitric oxide (NO) deficiency, is one of the causes of essential hypertension¹. Worldwide, hyper-tension is estimated to cause 7.1 million premature deaths per year and its prevalence in developing countries is already as high as those in developed countries². Nitric oxide (NO) is one of the smallest biologically active molecules that are produced from L-arginine by nitric oxide synthase (NOS). There are three iso forms of the nitric oxide synthase: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS)³. Chronic inhibition of NO synthesis by the administration of L-NAME (N^o-nitro-L arginine methyl ester) inhibits NOS activity and hence NO biosynthesis, leading to hypertension, atherosclerosis and cardiac remodeling⁴, an impairment of endothelial dependent relaxation and renal function alteration⁵. Experimental models of

hypertension have been associated with reduced serum and tissue level of magnesium (Mg), the second most abundant intracellular cation⁶. Mg is known to be essential for enzymatic activity, structural stability of anions such as nucleotides, and maintaining body physiology. Mg affects the electrical properties of membranes and their permeability characteristics⁷.

Plant polyphenolic compounds the flavanoids consist of number of classes, as flavanols, flavones and flavans. A naturally occurring flavones, Chrysin (5, 7-dihydroxy flavones structure shown in Fig. 1) contained in flowers blue passion flower (*Passiflora caerulea*), Indian trumpet flower, as well as in edible of mushrooms⁸, honey and propolis⁹. At the same time it possess antioxidant capacity, anti-inflammatory activity, anti-allergic, anti-cancer, antiestrogenic, anxiolytic¹⁰, antihypertensive properties¹¹. Chrysin having tyrosinase inhibitory activity, moderate aromatase inhibitory activity, and another important role are inhibits estradiol-induced DNA synthesis. C-iso-prenylated hydrophobic derivatives of chrysin are potential P-glycoprotein modulators in tumour cells¹². The earlier study showed that chrysin has antihypertensive effects, and reduces hepatic, renal damages and endothelial dysfunction in L-NAME induced hypertensive rats¹³. The present study aimed to evaluate the effect of chrysin on blood pressure, detection of

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Abbreviation: ACE, Angiotensin converting enzyme; L-NAME, N^o-nitro-L arginine methyl ester; Mg, magnesium; mRNA, m Ribosomal nitric acid; NOS, nitric oxide synthase

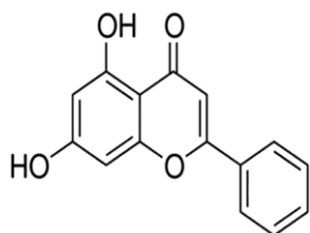


Fig. 1 — Chemical structure of chrysin (5,7 dihydroxyflavone)

mRNA expression of β -MHC and TGF- β , measurement of Mg levels in Plasma and fibrinogen, bilirubin and albumin in serum in the L-NAME induced hypertensive rats against the control and unsupplemented groups.

Materials and Methods

Chemicals

Chrysin and L-NAME was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade and obtained from E-Merck or HIMEDIA, Mumbai, India.

Animals

All the animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee of Bharathidasan University and animals were cared for in accordance with the Indian National Law on Animal Care and Use. Male Wistar rats (180-220 g) were purchased from the Indian Institute of Science, Bangalore, India. Rats were housed in plastic cages with filter tops under controlled conditions of a 12 h light-dark cycle, 50% humidity and temperature of 28°C. All rats received a standard pellet diet (Lipton Lever Mumbai, India) and water *ad libitum*.

Induction of L-NAME induced hypertension

L-NAME (40 mg/kg B.W) was dissolved in drinking water and given to rats at an interval of 24 h for 8 weeks. Mean arterial blood pressure (MAP) was measured using tail cuff method. MAP measurements were performed during the time of 1-8 weeks¹⁴.

Blood pressure measurements

Systolic and diastolic blood pressures were determined by the tail-cuff method (IITC, model 31, Woodland Hills, CA, USA). The animals were placed in a heated chamber at an ambient temperature of 30-34°C for 15 min and from each animal one to nine blood pressure values were recorded. The lowest three readings were averaged to obtain a mean blood pressure. All recordings and data analyses were done using a computerized data acquisition system and software.

Study design

Animals were divided into four groups of six rats each and all were fed the standard pellet diet. The rats were grouped as given below.

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|-----------|---|
| Group I | Control |
| Group II | Normal + Chrysin (25 mg/kg of B.W) after 4 th week |
| Group III | L-NAME induced hypertension (40 mg/kg of B.W) |
| Group IV | L-NAME induced hypertension (40 mg/kg of B.W) + Chrysin (25 mg/kg of B.W) |

Administered orally once in a day in the morning for 4 weeks. The compound was suspended in 2% dimethyl sulfoxide solution and fed group I and III by intubation. After 8 weeks morning, the animals were sacrificed by cervical dislocation. The blood was collected in clean dry test tubes and allowed to coagulate at ambient temperature for 30 min. Serum was separated by centrifugation at 2000 rpm for 10 min. The blood, collected in a heparinized centrifuge tube, was centrifuged at 2000 rpm for 10 min and the plasma separated was removed by aspiration and was used for estimations.

The Liver, Brain and kidney were immediately removed and washed in ice-cold saline to remove the blood. The tissues were sliced and homogenized in 0.1 M Tris-HCl buffer (pH 7.0). The homogenates were centrifuged at $48 \times g$ for 10 min at 4°C in a cold centrifuge. The supernatants were separated and used for the determination of various parameters.

Biochemical analysis

The serum fibrinogen¹⁵, albumin¹⁶ and serum bilirubin¹⁷ were measured.

Real-time polymerase chain reaction

Single-strand cDNA was reverse transcribed using a First Strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK) and gene-specific antisense primers (the Centre for Genomic Application, New Delhi, India). Primers for real-time reverse transcription polymerase chain reaction (RT-PCR) were designed for eNOS and cyclophilin A using Primer Express™ Software (PerkinElmer Inc., Waltham, MA, USA) and are listed in (Table 1). Real-time RT-PCR reactions were performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific) on ABI Prism 7300 Sequence Detection System (Thermo Fisher Scientific). For each 25 μ L of PCR reaction, 1.0 μ L of cDNA, 1.0 μ L of sense and antisense primers (2.5 μ M each), 12.5 μ L of SYBR Green PCR

Master Mix, and 10.5 μ L of PCR grade water were used. The cycling conditions were 94°C for 10 min, followed by 40 cycles of 94°C for 15 sec and 59°C for 30 sec. The PCR reactions were performed in five replicates for each gene. Relative transcript quantity was calculated using the $\Delta\Delta C_t$ method, with cyclophilin A as the endogenous reference gene amplified from the samples. A validation experiment for $\Delta\Delta C_t$ calculation was performed for each target by plotting the ΔC_t (C_t target – C_t reference) values versus log input amounts to create a semilog regression line. The slope of the resulting semilog regression line was used as a general criterion for the validation experiment. The absolute value of the slope was < 0.1 . Finally, the amount of target (drug treatment) normalized to the endogenous reference and relative to a calibrator (saline-treated control) was calculated by $2^{-\Delta\Delta C_t}$.²⁰

Statistical analysis

Statistical analysis were analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a commercially available Software Package for the Social Science (SPSS) software package version 11.0. Results were expressed as mean \pm SD for six rats in each group.

Table 1 — Details of primers used for real-time RT-PCR

Gene	Primer sequence
TGF- β	Forward primer: 5'-ATG ACA TGA ACC GAC CCT TC-3'
	Reverse primer : 5'-GTA GTT GGT ATC CAG GGC TCT C-3'
β -MHC	Forward primer: 5'-GTA GAC AAG GGC AAA GGC AA-3'
	Reverse primer : 5'-GGA TGA TGC AGC GTA CAA AG-3'
Cyclophilin A	5'-CAAACACAAATGGTTCCCAG-3'
	5'-ATGCTCATGCCTTCTTTACAC-3'
eNOS	5'-ACCGATACAACTACTTGAGGA-3'
	5'-AGCCACGTTAATTTCCACTG-3'

Notes: Primers were designed by Primer Express™ Software and were synthesized at The Centre for Genomic Application, New Delhi, India.

For all the statistical tests, values of $P < 0.05$ were statistically significant.

Results

Chrysin (25 mg/kg of B.W) dose was taken for this study based on previous our studies. Table 2 shows the levels of fibrinogen, albumin and serum bilirubin in L-NAME hypertensive rats before and after treatment with chrysin and in normotensives. L-NAME induced hypertensive rats had an elevated level of fibrinogen and a lowered level of serum bilirubin when compared to normotensives. There was no significant difference observed in the levels of albumin. A significant decrease in plasma fibrinogen level and increase in serum bilirubin levels were observed after chrysin consumption. We did not receive any reports on or find any adverse effects on the experimental animals either during or after the treatment period of group I and II.

Table 3 shows L-NAME rat hearts showed significantly ($P < 0.05$) elevated mRNA expression of TGF- β and β -MHC, compared with control. chrysin treatment significantly ($P < 0.05$) suppressed the expression of above mRNAs in the L-NAME rat heart. But there is no significant difference between group I and II.

eNOS mRNA expression studies

eNOS mRNA expression (Fig. 2) was studied in aorta, heart, and kidney using cyclophilin A as the internal standard. During the treatment with L-NAME, eNOS mRNA expression decreased significantly in all the three organs, whereas it increased during the treatment with chrysin in L-NAME-induced hypertensive rats with chrysin showing better results in the kidney. In the kidney, no significant difference was observed between the mRNA levels of chrysin and the control group. There is no significant difference between group I and II.

There are no changes between group I and II. Chrysin (25 mg/kg of B.W) is an effective dose for all parameters significant effect in L-NAME induced rats as compared to control rats. Chrysin in normal rats didn't show any significant.

Table 2 — Effect of chrysin on fibrinogen, bilirubin and albumin in serum of control and L-NAME induced hypertensive rats

	Control	Control+25 mg chrysin	L-NAME induced hypertension	L-NAME+25 mg chrysin
Fibrinogen (mg/dL)	216.02 \pm 14.48 ^a	215.95 \pm 13.62 ^a	327.85 \pm 19.39 ^b	219.72 \pm 15.27 ^c
Bilirubin(mg/dL)	0.95 \pm 0.19 ^a	0.96 \pm 0.08 ^a	0.72 \pm 0.06 ^b	0.94 \pm 0.07 ^c
Albumin (mg/dL)	4.82 \pm 0.08 ^a	4.83 \pm 0.05 ^a	4.70 \pm 0.04 ^b	4.09 \pm 0.07 ^c

Each value is mean \pm S.D. for six rats in each group.

Values not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT).

Table 3 — Effect of chrysin on mRNA expression of β -MHC and TGF- β in heart of control and L-NAME induced hypertensive rats

	Control	Control+25 mg chrysin	L-NAME induced hypertension	L-NAME+25 mg chrysin
β -MHC (Relative fold changes)	1.02 ± 0.05^a	1.02 ± 0.04^a	5.27 ± 0.07^b	1.05 ± 0.05^c
TGF- β (Relative fold changes)	1.03 ± 0.09^a	1.02 ± 0.08^a	5.99 ± 0.41^b	1.09 ± 0.07^c

Each value is mean \pm S.D. for six rats in each group.

Values not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT).

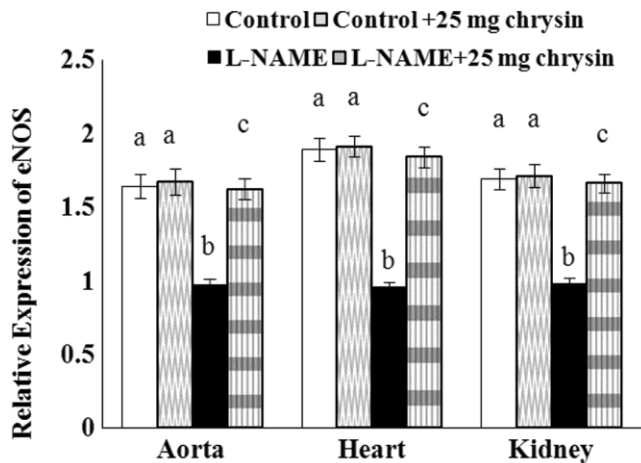


Fig. 2 — eNOS mRNA expression in aorta, heart, and kidney of the groups. Columns are mean \pm SD of six rats from each group. Columns not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT)

Discussion

Numerous cross-sectional epidemiologic studies¹⁸ have reported a modest positive association between plasma fibrinogen levels and blood pressure. Perhaps augmented synthesis of fibrinogen may be due to increased utilization during mechanical injury caused by L-NAME hypertension. Some experimental evidence suggests that administering pharmacologic agents to lower blood pressure can reduce fibrinogen levels or blood viscosity¹⁹; this also implies that high blood pressure may raise hemorrheologic parameters, rather than vice versa. Plasma fibrinogen concentration depends on the rate at which it is secreted into the blood from the liver where it is synthesized and on the rate of its elimination from the blood. This is either through the removal of intact fibrinogen by different organs; by its degradation in the blood to soluble products; or by conversion into and deposition as insoluble fibrin²⁰. A significant reduction in fibrinogen level was observed in patients with hypertension after chrysin supplementation. Probably, chrysin may facilitate uptake and degradation of plasma fibrinogen by the organs, thereby decreasing the plasma fibrinogen.

Bilirubin, a bile pigment, acts as a potent physiologic antioxidant that may provide important protection against coronary artery disease²¹. The lower serum bilirubin levels in the untreated L-NAME induced hypertensive rats to support the evidence of increased oxidant load in these rats²². The significant increase of serum bilirubin level in the supplementation with the increased antioxidants level we reported patients with L-NAME induced hypertensive rats in our study was compatible previously²³, from the chrysin. The plasma albumin concentration is a result of its rates of synthesis and degradation/excretion and its distribution between intra- and extravascular compartments²⁴. There was no significant difference observed in the level of albumin in all groups.

NO, essential for the proper functioning of cardiovascular system, is derived from L-arginine by NO synthase in endothelial cells. NO synthase inhibition produces hypertension, endothelial damage, cardiachypertrophy, inflammation, atherosclerosis, ventricular contractile dysfunction, and fibrosis²⁵. Chronic inhibition of NOS by L-NAME caused an increase of blood pressure associated with vascular structural change. It has been shown that the ventricular hypertrophy in L-NAME model of hypertension is linked to an increase of fibrosis and left ventricle hypertrophy.

Decreased functional activity of NOS in the long-term L-NAME-treated rats is well established. chrysin almost homogeneously increased the levels of eNOS mRNA expression in the tissues of the L-NAME-induced hypertension model, which was contradictory to the effect of L-NAME. These findings are in good agreement with the previously reported ACE inhibitors²⁶. Additionally, our present findings were also validated by the potency standard compound chrysin to restore eNOS mRNA expression in rat aorta, heart, and kidney with the effect being more prominent in kidney tissues.

We found that treatment with chrysin augmented eNOS expression. Such an increase in the expression

of eNOS might in turn have led to an increase in eNOS activity and NO production. Moreover, it is thought that this increase in the expression of eNOS might elicit the anti-inflammatory and antiatherosclerotic effects of a chrysin in the L-NAME- treated rat aorta. It should be noted this putative mechanism is also indicated by evidence demonstrating that chrysin improves endothelial dysfunction by increasing NO production in the hypertensive rat aorta, heart, and kidney.

At the molecular level, pathological stresses induce multiple changes, including genetic reprogramming (the re-expression of a battery of fetal genes and the down regulation of multiple adult genes). Together, these changes in gene expression result in substantial phenotypic changes, including changes in size, contractility, metabolic state, and electric conductance²⁷. The up-regulated gene TGF- β is a locally generated cytokine that has been implicated as a major contributor to tissue fibrosis and the latest studies in humans and experimental models have shown increased myocardial TGF- β expression during cardiac hypertrophy and fibrosis²⁸. Moreover, cardiac oxidative stress promotes the development of cardiac fibrosis by up-regulating TGF- β expression, which subsequently enhances cardiac collagen synthesis and suppresses collagen degradation in hypertensive rats²⁹. In the pressure-overloaded human heart, the up-regulation of ACE and TGF- β correlated with the degree of fibrosis³⁰. A recent study investigated the intracellular signalling events that are required for Ang II-dependent up-regulation of TGF- β revealed that the induction of TGF- β mRNA by Ang II in adult ventricular cardiomyocytes is mediated by NAD(P)H oxidase³¹. This indicates the possible role of oxidative stress in the TGF- β -Ang II (ACE) circuit and hypertrophy. In this study, the intervention of this circuit by chrysin (antioxidant role) at the level of oxidative stress and ACE prevents fibrosis and cardiac remodeling.

Pathological hypertrophy is characterized by increase in cardiomyocyte diameter, oxidative stress, fibrosis, and expression of β -MHC (a fetal specific isoform)³². The up-regulation of β -MHC transcription has been used as an early and sensitive marker of cardiac hypertrophy. In general, α -MHC is normally a predominant isoform occurring in the heart and expression of β -MHC contributes to the overall poor functioning of the hypertrophic ventricle³³. A previous study indicated that, oxidative stress induced by chronic hyperglycemia increased the activation of the pleiotropic transcription factor nuclear factor- κ B (NF- κ B). NF- κ B activation triggered a cascade of signalling, which finally led to

the switch in the cardiac MHC gene expression from the α -MHC isoform to the β -MHC isoform³⁴. Thus, the effect of chrysin on reduced expression of β -MHC isoform might be due to its preventive effect on oxidative stress which in turn suppresses the cardiac remodeling in L-NAME rats.

Conclusion

Our result explored that, oral administration of chrysin (25 mg/kg BWT) significantly decreased the expression of β -MHC and TGF- β and attenuated eNOS mRNA expression in L-NAME induced hypertensive rats. Thus, chrysin analogs might be potential therapeutic agents for treating L-NAME induced hypertension.

Conflict of interest

All authors declare no conflict of interest.

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